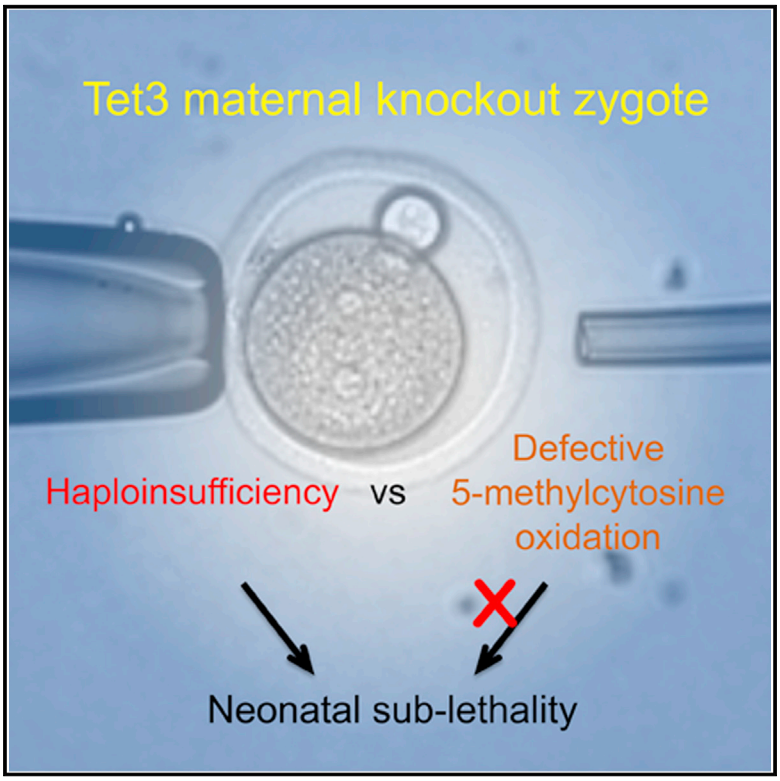
Haploinsufficiency, but Not Defective Paternal 5mC Oxidation, Accounts for the Developmental Defects of Maternal Tet3 Knockouts

Report

Cell Reports

Haploinsufficiency, but Not Defective Paternal 5mC Oxidation, Accounts for the Developmental Defects of Maternal Tet3 Knockouts

Graphical Abstract



Authors

Azusa Inoue, Li Shen, Shogo Matoba, Yi Zhang

Correspondence

yzhang@genetics.med.harvard.edu

In Brief

Using pronuclear transfer of Tet3 maternal KO zygotes, Inoue et al. demonstrate that sublethality of Tet3 maternal KO mice is due to haploinsufficiency rather than defective paternal 5mC oxidation.

Highlights

- Tet3 maternal KO causes neonatal sublethality
- The sublethality is caused by Tet3 haploinsufficiency
- Tet3-mediated paternal 5mC oxidation is dispensable for mouse development
- Paternal genome of Tet3 maternal KO embryos is hypomethylated by the blastocyst stage

Accession Numbers

GSE62719

Inoue et al., 2015, Cell Reports *10*, 463–470
February 3, 2015
http://dx.doi.org/10.1016/j.celrep.2014.12.049

Cell Reports
Report

Haploinsufficiency, but Not Defective Paternal 5mC Oxidation, Accounts for the Developmental Defects of Maternal Tet3 Knockouts

Azusa Inoue,[1,2,3] Li Shen,[1,2,3] Shogo Matoba,[1,2,3] and Yi Zhang[1,2,3,4,*]
[1]Howard Hughes Medical Institute, Harvard Medical School, WAB-149G, 200 Longwood Avenue, Boston, MA 02115, USA
[2]Program in Cellular and Molecular Medicine, Boston Children's Hospital, Harvard Medical School, WAB-149G, 200 Longwood Avenue, Boston, MA 02115, USA
[3]Department of Genetics, Harvard Medical School, WAB-149G, 200 Longwood Avenue, Boston, MA 02115, USA
[4]Harvard Stem Cell Institute, Harvard Medical School, WAB-149G, 200 Longwood Avenue, Boston, MA 02115, USA
*Correspondence: yzhang@genetics.med.harvard.edu
http://dx.doi.org/10.1016/j.celrep.2014.12.049

SUMMARY

Paternal DNA demethylation in mammalian zygotes is achieved through Tet3-mediated iterative oxidation of 5-methylcytosine (5mC) coupled with replication-dependent dilution. Tet3-mediated paternal DNA demethylation is believed to play important roles in mouse development given that *Tet3* heterozygous embryos derived from Tet3-deficient oocytes exhibit embryonic sublethality. Here, we demonstrate that the sublethality phenotype of the *Tet3* maternal knockout mice is caused by haploinsufficiency but not defective paternal 5mC oxidation. We found that *Tet3* heterozygous progenies derived from heterozygous father or mother also exhibit sublethality. Importantly, wild-type embryos reconstituted with paternal pronuclei that bypassed 5mC oxidation develop and grow to adulthood normally. Genome-scale DNA methylation analysis demonstrated that hypermethylation in maternal *Tet3* knockout embryos is largely diminished by the blastocyst stage. Our study thus reveals that Tet3-mediated paternal 5mC oxidation is dispensable for mouse development and suggests the existence of a compensatory mechanism for defective 5mC oxidation in preimplantation embryos.

INTRODUCTION

DNA methylation, the addition of a methyl group to the fifth position of cytosine (5-methylcytosine, 5mC), plays important roles in gene silencing and genome stability, and is essential for mammalian development (Smith and Meissner, 2013). DNA methylation is established by the de novo DNA methyltransferases DNMT3A and DNMT3B, and is maintained by DNMT1. Although the DNA methylation pattern is faithfully maintained throughout generations in somatic cells, it is globally erased during preimplantation development (Saitou et al., 2012; Sasaki and Matsui, 2008). After fertilization, both paternal and maternal genomes become hypomethylated and reach their lowest levels at the blastocyst stage even though the hypomethylated status is established differentially between the parental genomes. Maternal 5mC is mostly diluted in a DNA replication-dependent manner (Rougier et al., 1998), likely because of the limited availability of DNMT1 in early embryos (Hirasawa et al., 2008). In contrast, the paternal genome is subjected to global active demethylation (Mayer et al., 2000; Oswald et al., 2000). We and others have found that Tet3 oxidizes paternal 5mC into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine, and 5-carboxylcytosine in mouse zygotes (Gu et al., 2011; Inoue et al., 2011; Wossidlo et al., 2011), and that the 5mC oxidation products are gradually lost during preimplantation development through DNA replication-dependent passive dilution (Inoue et al., 2011; Inoue and Zhang, 2011). Recent genome-scale analyses have also revealed that a large proportion of paternal 5mCs undergo passive dilution without oxidation (Guo et al., 2014a; Shen et al., 2014).

In contrast to the great progress that has been made in understanding the mechanism of paternal DNA demethylation, the biological significance of this process remains poorly understood. The importance of this event for mammalian development was suggested by previous studies that demonstrated that paternal DNA demethylation is conserved in certain species, including rabbit, bovine, and human (Guo et al., 2014b; Lepikhov et al., 2008; Reis Silva et al., 2011; Wossidlo et al., 2011). However, a similar phenomenon has not been observed in sheep, pig, and goat, raising a question as to whether this event plays a general role in mammalian development (Beaujean et al., 2004; Jeong et al., 2007; Park et al., 2010). In addition, a previous study demonstrated that although paternal DNA demethylation was impaired in mouse zygotes derived from round spermatid injection, the development of the zygotes was normal (Polanski et al., 2008). Taken together, these studies have generated conflicting interpretations regarding the role of paternal DNA demethylation in mammalian development.

Nevertheless, it is generally believed that Tet3-mediated paternal DNA demethylation plays an important role in mouse

development (Kohli and Zhang, 2013; Messerschmidt et al., 2014; Pastor et al., 2013; Seisenberger et al., 2013; Wu and Zhang, 2014). This notion is based on a previous study in which it was observed that loss of maternal Tet3 protein prevents paternal 5mC oxidation in zygotes and leads to embryonic sublethality (Gu et al., 2011). In that study, heterozygous (Het) embryos derived from crosses of germ cell conditional knockout (CKO) females with wild-type (WT) males exhibited delayed expression of *Oct4* gene from the paternal allele during preimplantation development, and, importantly, ~40% of the Het embryos degenerated after midgestation (Gu et al., 2011). There are at least two possible explanations for this sublethal phenotype: First, Tet3-mediated paternal DNA demethylation is required for mouse development. However, delayed paternal *Oct4* expression is unlikely to be the cause of the sublethality because it is recovered by the blastocyst-stage, a stage beyond which the embryos can develop normally (Gu et al., 2011). The second possibility is *Tet3* haploinsufficiency due to deletion of the maternal allele. This possibility should be considered given that 42% of previously screened mouse genes exhibit haploinsufficiency (White et al., 2013). Thus, it is necessary to evaluate these two possibilities to determine whether Tet3-mediated paternal DNA demethylation is required for mouse development.

In this study, we demonstrated that the sublethality of *Tet3* maternal KO mice is caused by *Tet3* haploinsufficiency, but not by defective paternal 5mC oxidation. Furthermore, genome-scale DNA methylation analysis revealed that hypermethylation in maternal KO zygotes is largely reset by the blastocyst stage, suggesting the existence of a compensatory demethylation pathway in preimplantation embryos.

RESULTS

Tet3 Maternal KO Causes Neonatal Sublethality

As reported recently (Shen et al., 2014), we generated a *Tet3* conditional KO (CKO) mouse that allows deletion of Tet3 in oocytes expressing $Zp3^{Cre}$. We refer to two loxP and one loxP (deleted) alleles as "f" and "–," respectively. Immunostaining with an anti-Tet3 antibody confirmed that Tet3 is depleted in zygotes derived from oocytes of [$Zp3^{Cre}$, $Tet3^{f/f}$] females fertilized with WT sperm (maternal KO zygotes). In contrast, Tet3 is readily detectable in the paternal pronuclei of control zygotes derived from oocytes of [$Tet3^{f/f}$] females (Figure 1A). Consistent with the notion that 5hmC in paternal pronuclei is generated by maternally deposited Tet3, 5hmC is completely lost in maternal KO zygotes, whereas the 5mC signal in paternal pronuclei is slightly increased (Figure 1B).

To examine the developmental potential of maternal KO embryos, we crossed WT [$Tet3^{f/f}$] or CKO [$Zp3^{Cre}$, $Tet3^{f/f}$] females with WT males. Caesarian section (C-section) at embryonic day 19.5 (E19.5) revealed that the maternal KO embryos did not show a significant increase of developmental failure as evidenced by implantation sites and dead bodies (Figure 1C; Table S1). We confirmed that all of the maternal KO embryos genotyped were Het (29/29), indicating efficient deletion of the Tet3 allele by $Zp3^{Cre}$. This result indicates that defective paternal 5mC oxidation does not significantly compromise embryonic development. This seems to be in conflict with a previous report that ~40% of Tet3 maternal KO embryos ([$TNAP^{Cre}$, $Tet3^{f/–}$] female × WT male) die before birth (Gu et al., 2011). This phenotypic difference could be caused by the different maternal genotypes employed in the two studies, as we used CKO mice with a WT background ([$Tet3^{f/f}$]), whereas the previous study used mice with a Het background ([$Tet3^{f/–}$]). To test this possibility, we generated CKO females with a Het background [$Zp3^{Cre}$, $Tet3^{f/–}$] and crossed with WT males. C-section at E19.5 revealed that, consistent with the previous report, ~40% of embryos showed lethality during embryogenesis (Figure 1C; Table S1).

We next assessed the neonatal and postnatal growth of the maternal KO progenies that were derived from [$Zp3^{Cre}$, $Tet3^{f/f}$] females naturally mated with WT males. Daily checking of delivery and counting the number of surviving pups revealed that a significantly larger population (33%) of maternal KO progenies died within 3 days after birth compared with controls (17%) (Figure 1D; Table S2). The surviving progenies at day 3 were viable and grew normally (Figure 1E). Thus, these results indicate that *Tet3* maternal KO progenies display neonatal sublethality.

Tet3 Heterozygous Mice Exhibit Neonatal Sublethality

Because maternal KO progenies are Het, and Tet3 null mice are known to be neonatal lethal (Gu et al., 2011; Wang et al., 2013), the observed sublethality could be caused by *Tet3* haploinsufficiency. To examine this possibility, we asked whether *Tet3* Het progenies derived from Het females exhibit neonatal sublethality similarly to the maternal KO progenies. Mating of Het females with WT males followed by C-section at E19.5 showed no significant developmental failure in the embryos (Figure 2A; Table S3). As expected, about half (49%) of the embryos were Het (Figure 2B). Analysis of neonatal and postnatal growth indicated that a significant population (31%) of the progenies died within 3 days after birth (Figure 2C; Table S4). All of the progenies that survived the first 3 days were viable at least until 20 days after birth. Genotyping of the living pups at 20 days after birth revealed that the Het population (38%) was markedly smaller than the WT population (Figure 2D). These results indicate that *Tet3* Het progenies exhibit neonatal sublethality in a similar fashion to maternal KO progenies.

It is assumed that oocytes from Het females may have only half the amount of Tet3 protein, which may cause incomplete oxidation of paternal 5mC in zygotes. This assumption makes it unclear whether the sublethality of *Tet3* Het progenies is caused by haploinsufficiency at the neonatal stage or by a potential compromise of paternal 5mC oxidation at the zygote stage. To distinguish between these possibilities, we generated *Tet3* Het males and crossed them with WT females. Given that paternal 5mC oxidation is solely dependent on maternally deposited Tet3 protein (Gu et al., 2011, Guo et al., 2014a; Shen et al., 2014), zygotes derived from these mating pairs should undergo normal 5mC oxidation. Notably, natural mating of Het males with WT females also resulted in neonatal sublethality (Figure 2C; Table S4). Genotyping of the surviving pups at 20 days after birth revealed that the Het population (34%) was substantially smaller than the WT population (Figure 2D), similar to what was observed in crosses of Het females with WT males. Taken together, these results demonstrate that *Tet3* Het mice exhibit haploinsufficiency defects in neonatal development.

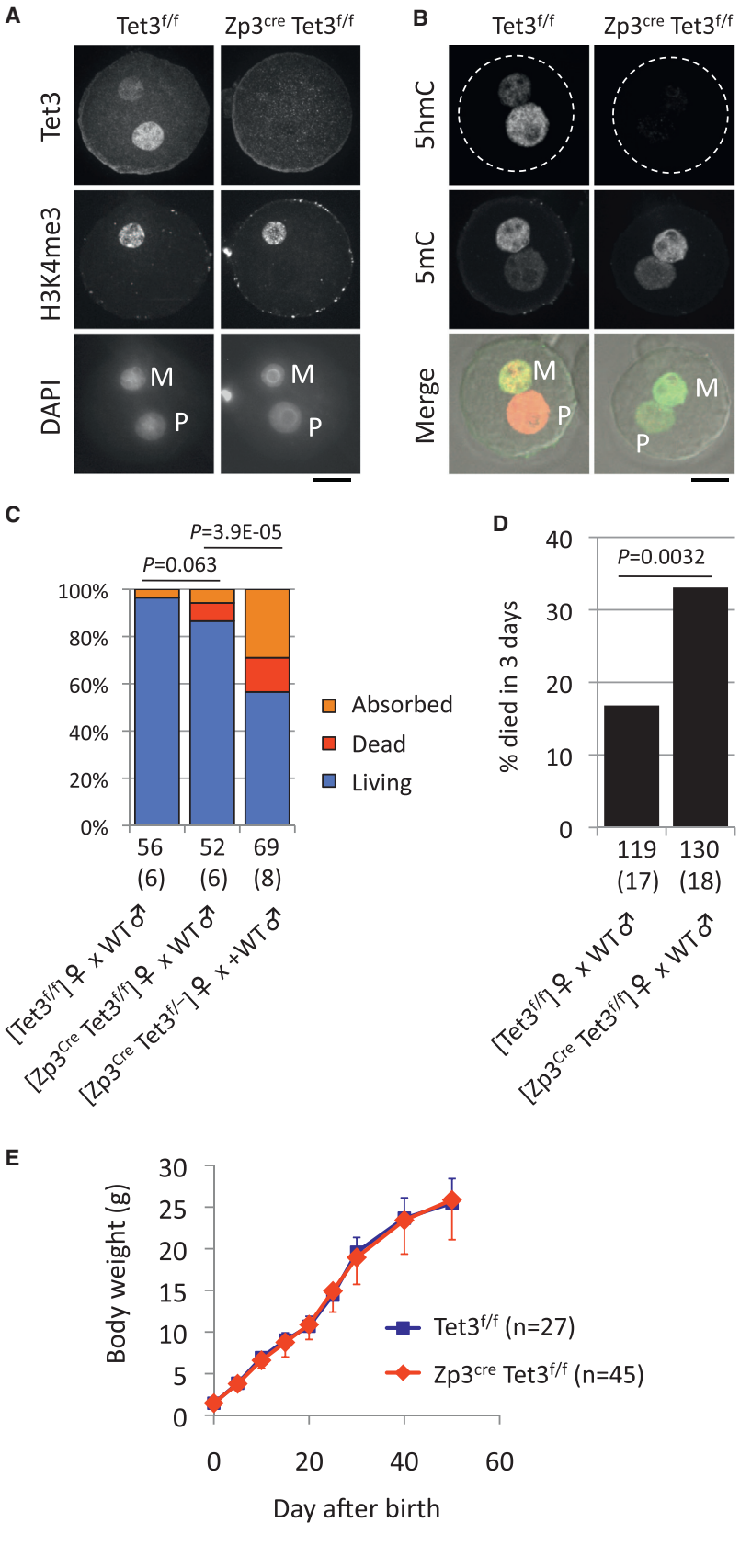


Figure 1. *Tet3* Maternal KO Progenies Show Neonatal Sublethality

(A) Representative images of zygotes stained with anti-Tet3 antibody. Maternal chromatin is marked by H3K4me3. M, maternal pronucleus; P, paternal pronucleus. Scale bar represents 20 μm.

(B) Representative images of zygotes stained with anti-5hmC (red in merge) and anti-5mC (green in merge).

(C) Percentage of living (blue), dead (red), and absorbed (orange) embryos at E19.5. Implantation sites without visible embryos were counted as "absorbed." Dead bodies at birth were counted as "dead." C57BL/6J males were used for mating. The total number of embryos obtained is indicated below the bars. The total number of litters examined is indicated within the parentheses. p, χ^2 test for "living." See also Table S1.

(D) Percentage of progenies that died within 3 days after birth. C57BL/6J males were used for mating. The total number of progenies examined is indicated below the bars. The total number of deliveries during mating term for 3–4 months is indicated within parentheses. A total of seven mating pairs were examined in both groups. p, χ^2 test. See also Table S2.

(E) Growth curve of progenies from the indicated females. The number of progenies examined is indicated in parentheses. Error bar represents SD.

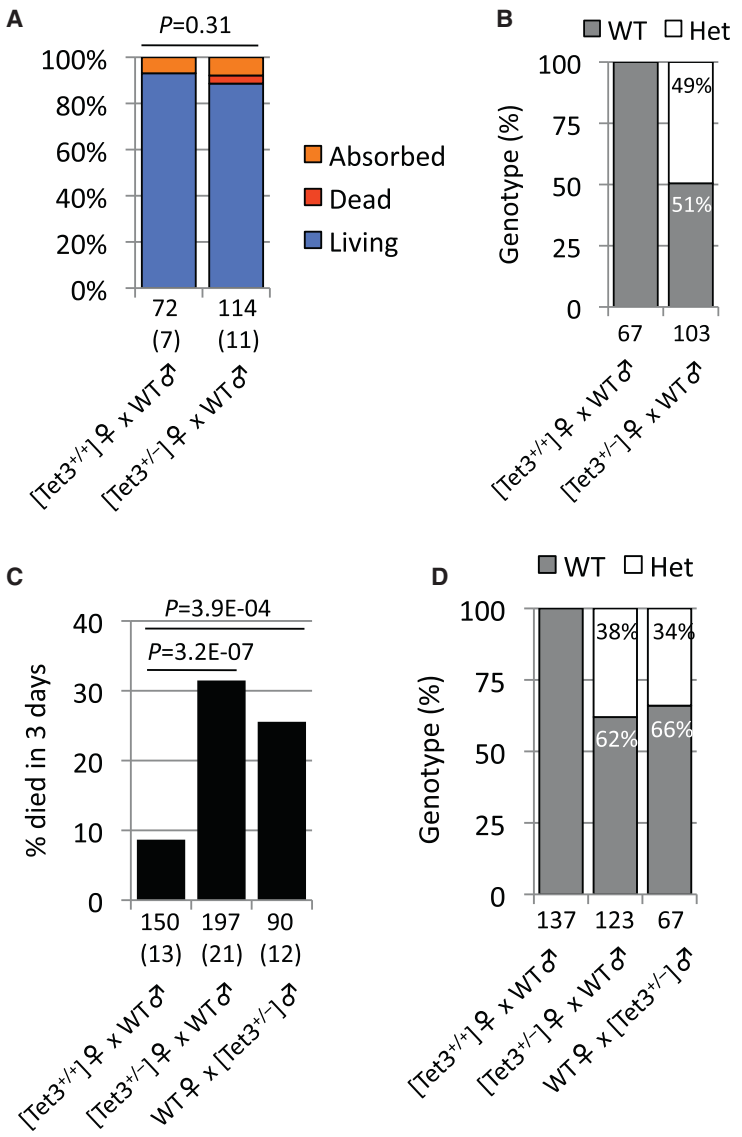


Figure 2. *Tet3* Heterozygous Mice Show Neonatal Sublethality

(A) Percentage of living (blue), dead (red), and absorbed (orange) embryos at E19.5. Implantation sites without visible embryos were counted as "absorbed." Dead bodies at birth were counted as "dead." C57BL/6J males were used for mating. The total number of embryos obtained is indicated below the bars. The total number of litters examined is indicated within parentheses. *p*, χ^2 test for "living." See also Table S3.

(B) Genotype of embryos living at E19.5. The total number of pups examined is indicated below the bars.

(C) Percentage of progenies that died within 3 days after birth. C57BL/6J males were used for mating as WT. The total number of progenies examined is indicated below the bars. The total number of deliveries during the mating term for 3–4 months is indicated within parentheses. A total of five, nine, and four mating pairs were examined for [*Tet3*$^{+/+}$] females, [*Tet3*$^{+/-}$] females, and [*Tet3*$^{+/-}$] males, respectively. p, χ^2 test. See also Table S4.

(D) Genotype of living pups at 20 days after birth. The total number of pups examined is indicated below the bars.

Tet3-Mediated Paternal 5mC Oxidation Is Dispensable for Mouse Development

Although the above results suggest that the *Tet3* gene shows haploinsufficiency, it is still unclear whether Tet3-mediated paternal 5mC oxidation is developmentally important. Thus, it is necessary to distinguish the potential effect of defective paternal 5mC oxidation from the effect of haploinsufficiency. Therefore, we attempted to reconstruct genetically WT zygotes that bypass paternal 5mC oxidation through pronuclear transfer (NT). First, we removed paternal pronuclei from WT zygotes at the late-zygotic (PN5) stage and used the remaining cytoplasm containing maternal pronuclei as recipients (Figure 3A). We then isolated paternal pronuclei from maternal KO zygotes ([*Zp3*^{Cre}, *Tet3*$^{f/f}$] females × WT males) at the same zygotic stage as donor pronuclei. Importantly, although the donor paternal pronuclei were genetically WT, they escaped Tet3-mediated 5mC oxidation. Fusion with the recipients allowed us to reconstruct genetically WT zygotes with paternal pronuclei, bypassing 5mC oxidation (Figure 3A, NT-KO). As a control, we used paternal pronuclei from WT zygotes ([*Tet3*$^{f/f}$] females × WT males) that had gone through 5mC oxidation as donors (Figure 3A, NT-WT).

Immunostaining with anti-5hmC antibody at the two-cell stage (20 hr after NT) confirmed that the 5hmC levels in NT-KO embryos were as low as those in maternal KO embryos, indicating that Tet3-mediated paternal 5mC oxidation does not occur in NT-KO embryos (Figure 3B). This suggests that Tet3 protein retained in the recipient cytoplasm is not sufficient to trigger massive 5mC oxidation in the reconstructed embryos. This is plausible because Tet3 mainly localizes to the paternal pronuclei that have been removed from the recipients (Gu et al., 2011; Inoue et al., 2012). Additionally, since Tet3 is no longer localized to nuclei after the first mitosis (Gu et al., 2011), the time window during which the remaining Tet3 can function is very limited due to the quick entry of the embryo into the first mitosis (within 2 hr after fusion). Thus, we successfully created genetically WT zygotes without paternal 5mC oxidation.

To examine the development of the reconstructed embryos, we transplanted them into pseudopregnant females. C-section at E19.5 revealed that NT-KO embryos could develop to term at a ratio similar to that observed for NT-WT embryos (Figures 3C and 3D; Table S5). Furthermore, no significant differences in the weights of embryos and placentae were observed (Figure 3E). Importantly, no NT-KO pups showed neonatal lethality

and all of the mice developed normally to adulthood (Figures 3F and 3G). Mating of adult NT-KO mice with WT mice confirmed that both NT-KO males and females were fertile (Figure 3H). Taken together, these results demonstrate that Tet3-mediated paternal 5mC oxidation is dispensable for mouse development.

The Paternal Genome of Maternal *Tet3* KO Embryos Is Hypomethylated by the Blastocyst Stage

After the first wave of DNA demethylation in zygotes, the embryonic genome becomes further demethylated during preimplantation development and reaches its lowest point of methylation at the blastocyst stage (Guo et al., 2014b; Smith et al., 2012, 2014). To examine the effect of the maternal loss of Tet3 on DNA methylation at the blastocyst stage, we performed a genome-scale methylome analysis on one-cell and blastocyst-stage embryos generated using *Tet3* CKO oocytes (C57BL/6J × 129/Sv background) and CAST/EiJ sperm (Table S6). Due to the limited cell numbers of the samples, we used the reduced representative bisulfite sequencing (RRBS) method, which samples ~5% of total CpG of the mouse genome (Smith et al., 2012). Using SNP information unique to the CAST strain, we dissociated the methylation state of the paternal genome from that of the maternal genome. In total, we identified 83,172 SNP-tracked CpGs commonly covered in all samples, among which 17,442 CpGs undergo dramatic DNA demethylation in zygotes (methylation level in sperm [ML_{sp}] $\geq$ 80%, and relative demethylation level in WT zygotes [RDL_{WT}] $\geq$ 0.3, where RDL_{WT} is defined as [$ML_{Sp} - ML_{WT}$]/ML_{Sp}). We then focused on Tet3-dependent demethylated CpGs, which showed lower RDL values in maternal KO zygotes than in WT ($RDL_{CKO}/RDL_{WT} \leq 0.6$, n = 10,559), and then examined the MLs of blastocyst embryos (Figure 4A). Interestingly, the hypermethylated CpG sites in maternal KO zygotes became drastically hypomethylated at the blastocyst stage, and the extent of the methylation difference between WT and maternal KO was much less obvious (Figures 4A and 4B), suggesting that demethylation of these loci takes place independently of maternal Tet3. These results imply that even in the absence of Tet3-mediated oxidation at zygotes, the paternal genome can be globally hypomethylated by the blastocyst stage, which may explain why Tet3-mediated paternal 5mC oxidation is dispensable for later development.

DISCUSSION

Based on the previous report that maternal depletion of Tet3 blocks paternal 5mC oxidation and leads to embryonic sublethality (Gu et al., 2011), it has been believed that Tet3-mediated paternal 5mC oxidation plays an important role in mouse development (Kohli and Zhang, 2013; Messerschmidt et al., 2014; Pastor et al., 2013; Seisenberger et al., 2013; Wu and Zhang, 2014). However, because the progenies of *Tet3* CKO females are heterozygous, it remained to be determined whether the sublethality was due to *Tet3* haploinsufficiency or defective paternal 5mC oxidation. In this study, we explored these two possibilities and found that haploinsufficiency, but not defective paternal 5mC oxidation, is the cause of this phenotype. The notion that proper expression of *Tet3* is required for neonatal growth is consistent with previous findings that Tet3 null mice exhibit neonatal lethality (Gu et al., 2011; Wang et al., 2013). Future studies should reveal why Tet3 is required for neonatal growth.

The mammalian zygote is one of the best in vivo models for studying the mechanism of DNA demethylation. Interestingly, a recent study suggested that 5mCs within certain genomic loci are converted to unmodified cytosines in mouse zygotes in a Tet3-dependent but thymine DNA glycosylase (TDG)-independent manner, implying the existence of an undefined demethylation pathway (Guo et al., 2014a). To reveal such a mechanism, a hypothesis-driven candidate approach will be required because genome-wide screening is difficult to perform in zygotes (Gkountela and Clark, 2014). However, there may be many candidate factors, including deaminases, base excision repair enzymes, decarboxylases, and the elongator, that could be involved in active DNA demethylation (Messerschmidt et al., 2014; Wu and Zhang, 2014; Wu and Zhang, 2010). Since Tet3-mediated 5mC oxidation was thought to be required for development, only genes known to be relevant to development might have been listed as candidates. Nevertheless, our study indicates that such factors are not necessarily essential for development, and thus nonessential genes should also be considered.

We and others recently reported that Tet3 oxidizes not only the paternal genome but also the maternal genome, although to a lesser extent (Guo et al., 2014a; Shen et al., 2014). Because our NT experiment could not elucidate the role of maternal 5mC oxidation, we cannot exclude the possibility that Tet3-mediated maternal 5mC oxidation may play a role in development. Nevertheless, this possibility is less likely given that the phenotypes of maternal KO embryos, in which both paternal and maternal 5mC oxidations are defective, are not more severe than those of the Het embryos, in which 5mC oxidation takes place in both genomes normally, derived from Het males or females crossed with WT (compare Figures 1D and 2C).

We found that the paternal genome can be largely hypomethylated by the blastocyst stage in the absence of maternal Tet3 (Figure 4). This demethylation might be achieved by DNA replication-dependent passive dilution of 5mC during preimplantation development. This notion is supported by our recent observation that Tet3-dependent demethylated regions can partially undergo replication-coupled demethylation at the one-cell stage (Shen et al., 2014). Thus, consecutive passive dilutions during preimplantation development might compensate for the loss of maternal Tet3, leading to hypomethylation of the paternal genome. Alternatively, Tet1 and Tet2 might also contribute to DNA demethylation, as both begin to be expressed after the two-cell stage (Iqbal et al., 2011). It is also possible that a Tet-independent demethylation pathway is involved in the observed compensation (Wang et al., 2014). Further studies will be needed to identify all the players involved in DNA demethylation and to address the biological significance of global DNA demethylation in preimplantation embryos.

EXPERIMENTAL PROCEDURES

Mice

All animal studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at Harvard Medical School. *Tet3* CKO mice were generated as described previously (Shen et al., 2014). *Tet3* Het and WT mice were obtained by crossing CKO ([$Zp3^{Cre}$, $Tet3^{f/f}$] or

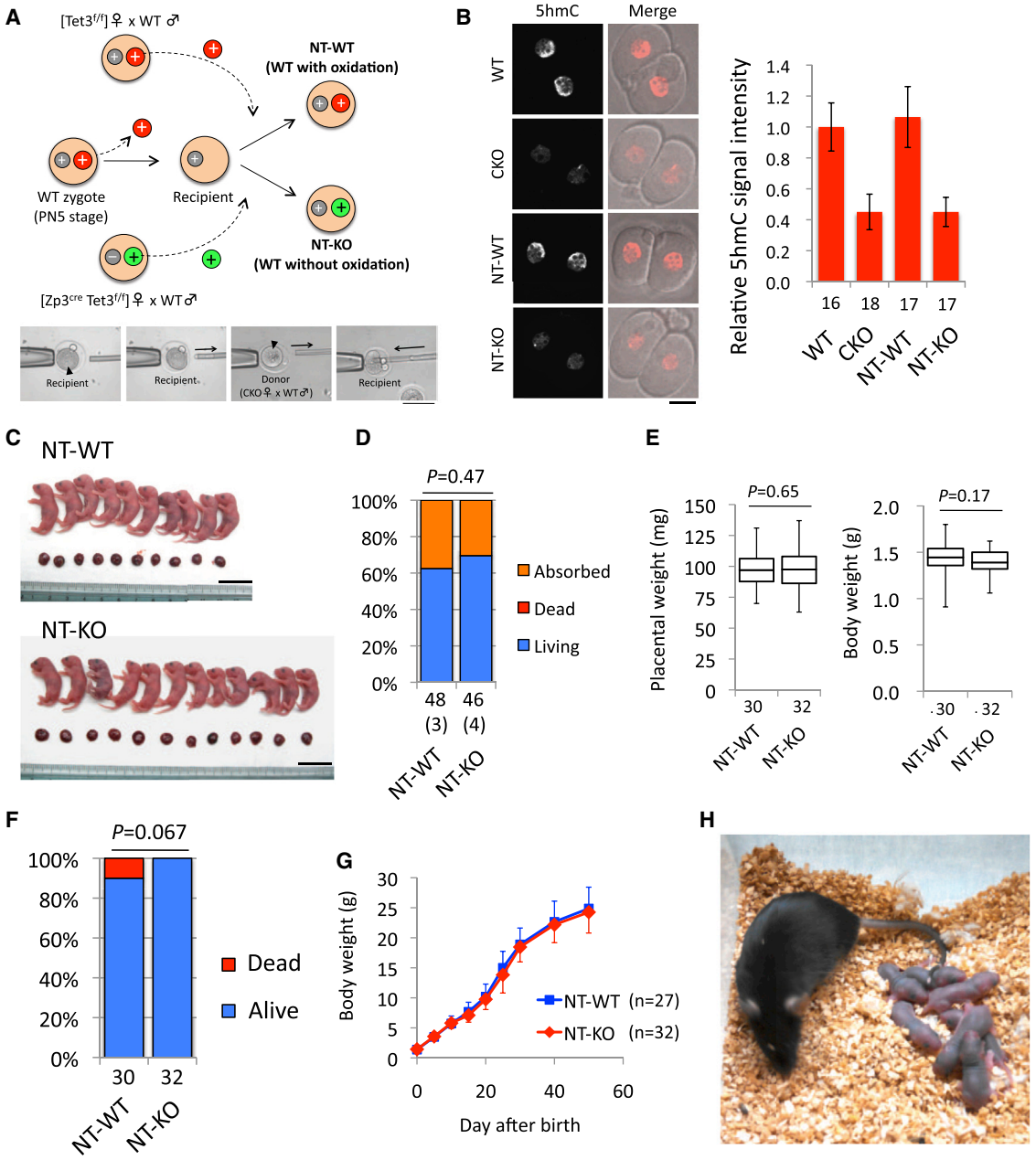


Figure 3. Tet3-Mediated Paternal 5mC Oxidation Is Dispensable for Mouse Development

(A) Schematic presentation of the pronuclear transfer (NT) procedure. The recipient cytoplasm was prepared by removing the paternal pronucleus from PN5-stage WT zygotes. Donor pronuclei were isolated from zygotes obtained from CKO [*Zp3*^{Cre}, *Tet3*$^{f/f}$] or WT [*Tet3*$^{f/f}$] females mated with WT males. Fusion with the recipients yields genetically WT zygotes with defective paternal 5mC oxidation (NT-KO) or control zygotes (NT-WT). Gray, maternal pronuclei; green, paternal pronuclei with defective 5mC oxidation; red, paternal pronuclei with oxidized 5mC; +, *Tet3* WT allele; –, *Tet3* KO allele. Images at bottom represent manipulation of NT. Black arrowheads indicate paternal pronuclei. Scale bar represents 100 μm.

(B) Representative images of two-cell embryos stained with anti-5hmC antibody. WT and CKO embryos were prepared as positive and negative controls, respectively. Scale bar represents 20 μm. The graph at right indicates quantification of the 5hmC signal. The value of WT embryos was set as 1.0. The number of embryos examined is indicated below the bars. Error bars, SD.

(C) Representative images of embryos and placentae from a single litter of NT-WT and NT-KO at E19.5. Scale bars represent 20 mm.

(D) Percentage of living (blue) and absorbed (orange) embryos at E19.5. Implantation sites without visible embryos were counted as "absorbed." Dead bodies were not observed in both groups. The total number of embryos examined is indicated below the bars. The total number of litters is indicated within parentheses. p, χ^2 test.

(legend continued on next page)

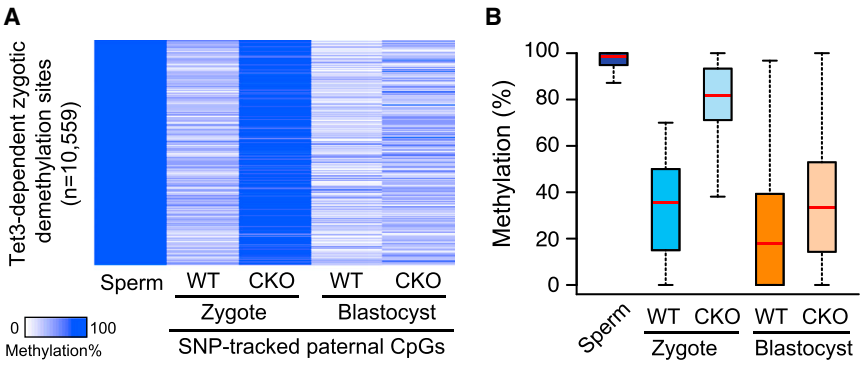


Figure 4. Effect of Maternal *Tet3* KO on the Paternal Methylome

(A) Heatmap of 10,559 SNP-tracked paternal CpG sites that are methylated in sperm ($ML_{Sp} \geq 80\%$) and demethylated after fertilization in a Tet3-dependent manner ($RDL_{WT\ Zygote} \geq 0.3$ and $RDL_{CKO\ Zygote}/RDL_{WT\ Zygote} \leq 0.6$). ML, methylation level; RDL, relative demethylation level, defined as $[(ML_{Sp} - ML_{WT\ Zygote})/ML_{Sp}]$. CKO, *Tet3* maternal KO.

(B) Box plot of MLs. The red line represents the median. Boxes and whiskers represent the 25th/75th and 2.5th/97.5th percentiles, respectively.

See also Table S6.

[$Zp3^{Cre}$, $Tet3^{f/-}$]) females with C57BL/6J males, followed by crosses of the Het progenies with C57BL/6J mice. The genotyping primer sequences were reported previously (Shen et al., 2014). The day when a vaginal plug appeared at noon was defined as E0.5. At E19.5, the progenies were collected by dissecting pregnant females that had been injected with 0.2 ml of 10 mg/ml progesterone (Sigma-Aldrich) at E17.5 and E18.5. For neonatal and postnatal growth, we took daily recordings of delivery and viability from natural mating pairs with C57BL/6J mice.

Pronuclear Transfer

For preparation of donor zygotes, 8-week-old CKO [$Zp3^{Cre}$, $Tet3^{f/f}$] or WT [$Tet3^{f/f}$] females were superovulated by injecting 7.5 I.U. of PSMG (Millipore) and hCG (Millipore) followed by mating with C57BL/6J males. For preparation of recipient zygotes, 8-week-old B6D2F1 females were superovulated similarly and mated with C57BL/6J males. At noon of day E0.5, PN2-3 zygotes were collected and cultured in KSOM (Millipore) in a humidified atmosphere of 5% CO_2/95% air at 37.8°C. Five hours later, zygotes reached the PN5 stage and were then transferred into M2 media containing 5 μM cytochalasin B (Sigma-Aldrich). Zona pellucidae were cut by a Piezo impact-driven micromanipulator (Prime Tech). The paternal pronuclei were removed from the recipient zygotes, and the remaining cytoplasms containing maternal pronuclei served as recipients. Parental pronuclei were distinguished by the distance from the second polar body and by the pronuclear size. Next, paternal pronuclei were isolated from PN5-stage WT or CKO zygotes and fused with the recipients by using sendai virus (HVJ; Cosmo-bio) as described previously (Inoue et al., 2008).

Two-cell-stage embryos were transferred to the oviducts of pseudopregnant (E0.5) ICR females. The pups were recovered by C-section on the day of delivery (E19.5) and nursed by lactating ICR females. After they grew to the adulthood, each of three NT-KO males and females were mated with C57BL/6J mice for fertility tests, and all gave 7–11 pups.

Immunostaining

Tet3, 5mC, and 5hmC staining was performed as described previously (Shen et al., 2014).

RRBS

MII oocytes were collected from 8-week-old superovulated females. They were transferred into HTF medium supplemented with 10 mg/ml BSA (Sigma-Aldrich) and inseminated with activated spermatozoa collected from the caudal epididymides of adult CAST/EiJ males. Five hours after fertilization, the zygotes were transferred into KSOM. Zygotes and blastocysts were collected at 13 and 96 hr postfertilization, respectively. Biological duplicates for each sample were collected, with each sample containing 40–50 zygotes or four blastocysts. Sperm genomic DNA was extracted from CAST males as described previously (Weyrich, 2012).

RRBS analyses were performed as described previously (Shen et al., 2014) and sequencing reads were mapped to the mouse genome (mm9) using Bismark v0.10.1 (Babraham Bioinformatics) after adaptor trimming by Trim Galore (Babraham Bioinformatics) with the "–rrbs" option. Paternal reads were extracted from the total mapped reads by SNPs between CAST and C57BL/6J mice. The ML of each covered cytosine in CpG context was calculated by dividing the number of reported C with the total number of reported C and T. Only CpG sites that were commonly covered by at least five reads in all samples were used for the subsequent analyses.

ACCESSION NUMBERS

The NCBI GEO accession number for the RRBS data reported in this paper is GSE62719.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.12.049.

AUTHOR CONTRIBUTIONS

A.I. and Y.Z. conceived the project, designed the experiments, and wrote the manuscript. A.I. performed the experiments. L.S. prepared the RRBS libraries and analyzed the sequencing data. S.M. assisted in some of the experiments.

ACKNOWLEDGMENTS

We thank L.M. Tuesta and S. Yamaguchi for critical readings of the manuscript and G.L. Xu for the Tet3 antibody. The work was partly supported by NIH grant U01-DK089565. S.M. is a research fellow for Research Abroad of the Japan Society for the Promotion of Science. Y.Z. is an investigator of the Howard Hughes Medical Institute.

(E) Box plot representations of body and placental weight. Middle lines in the boxes indicate the medians. Box edges and whiskers indicate the 25th/75th and 0th/100th percentiles, respectively. p, two-tailed Student's t test.

(F) Percentage of progenies that were alive (blue) or dead within 3 days after birth (red). The total number of progenies examined is indicated below the bars. p, χ^2 test.

(G) Growth curve of progenies. The number of pups examined is indicated in parentheses. Error bar represents SD.

(H) Representative image of an adult NT-KO female with its pups after crossing with a WT male.

See also Table S5.

Received: October 22, 2014
Revised: December 4, 2014
Accepted: December 19, 2014
Published: January 29, 2015

REFERENCES

Beaujean, N., Hartshorne, G., Cavilla, J., Taylor, J., Gardner, J., Wilmut, I., Meehan, R., and Young, L. (2004). Non-conservation of mammalian preimplantation methylation dynamics. Curr. Biol. *14*, R266–R267.

Gkountela, S., and Clark, A.T. (2014). A big surprise in the little zygote: the curious business of losing methylated cytosines. Cell Stem Cell *15*, 393–394.

Gu, T.P., Guo, F., Yang, H., Wu, H.P., Xu, G.F., Liu, W., Xie, Z.G., Shi, L., He, X., Jin, S.G., et al. (2011). The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature *477*, 606–610.

Guo, F., Li, X., Liang, D., Li, T., Zhu, P., Guo, H., Wu, X., Wen, L., Gu, T.P., Hu, B., et al. (2014a). Active and passive demethylation of male and female pronuclear DNA in the Mammalian zygote. Cell Stem Cell *15*, 447–458.

Guo, H., Zhu, P., Yan, L., Li, R., Hu, B., Lian, Y., Yan, J., Ren, X., Lin, S., Li, J., et al. (2014b). The DNA methylation landscape of human early embryos. Nature *511*, 606–610.

Hirasawa, R., Chiba, H., Kaneda, M., Tajima, S., Li, E., Jaenisch, R., and Sasaki, H. (2008). Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. Genes Dev. *22*, 1607–1616.

Inoue, A., and Zhang, Y. (2011). Replication-dependent loss of 5-hydroxymethylcytosine in mouse preimplantation embryos. Science *334*, 194.

Inoue, A., Nakajima, R., Nagata, M., and Aoki, F. (2008). Contribution of the oocyte nucleus and cytoplasm to the determination of meiotic and developmental competence in mice. Hum. Reprod. *23*, 1377–1384.

Inoue, A., Shen, L., Dai, Q., He, C., and Zhang, Y. (2011). Generation and replication-dependent dilution of 5fC and 5caC during mouse preimplantation development. Cell Res. *21*, 1670–1676.

Inoue, A., Matoba, S., and Zhang, Y. (2012). Transcriptional activation of transposable elements in mouse zygotes is independent of Tet3-mediated 5-methylcytosine oxidation. Cell Res. *22*, 1640–1649.

Iqbal, K., Jin, S.G., Pfeifer, G.P., and Szabó, P.E. (2011). Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. Proc. Natl. Acad. Sci. USA *108*, 3642–3647.

Jeong, Y.S., Yeo, S., Park, J.S., Koo, D.B., Chang, W.K., Lee, K.K., and Kang, Y.K. (2007). DNA methylation state is preserved in the sperm-derived pronucleus of the pig zygote. Int. J. Dev. Biol. *51*, 707–714.

Kohli, R.M., and Zhang, Y. (2013). TET enzymes, TDG and the dynamics of DNA demethylation. Nature *502*, 472–479.

Lepikhov, K., Zakhartchenko, V., Hao, R., Yang, F., Wrenzycki, C., Niemann, H., Wolf, E., and Walter, J. (2008). Evidence for conserved DNA and histone H3 methylation reprogramming in mouse, bovine and rabbit zygotes. Epigenetics Chromatin *1*, 8.

Mayer, W., Niveleau, A., Walter, J., Fundele, R., and Haaf, T. (2000). Demethylation of the zygotic paternal genome. Nature *403*, 501–502.

Messerschmidt, D.M., Knowles, B.B., and Solter, D. (2014). DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos. Genes Dev. *28*, 812–828.

Oswald, J., Engemann, S., Lane, N., Mayer, W., Olek, A., Fundele, R., Dean, W., Reik, W., and Walter, J. (2000). Active demethylation of the paternal genome in the mouse zygote. Curr. Biol. *10*, 475–478.

Park, J.S., Lee, D., Cho, S., Shin, S.T., and Kang, Y.K. (2010). Active loss of DNA methylation in two-cell stage goat embryos. Int. J. Dev. Biol. *54*, 1323–1328.

Pastor, W.A., Aravind, L., and Rao, A. (2013). TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. Nat. Rev. Mol. Cell Biol. *14*, 341–356.

Polanski, Z., Motosugi, N., Tsurumi, C., Hiiragi, T., and Hoffmann, S. (2008). Hypomethylation of paternal DNA in the late mouse zygote is not essential for development. Int. J. Dev. Biol. *52*, 295–298.

Reis Silva, A.R., Adenot, P., Daniel, N., Archilla, C., Peynot, N., Lucci, C.M., Beaujean, N., and Duranthon, V. (2011). Dynamics of DNA methylation levels in maternal and paternal rabbit genomes after fertilization. Epigenetics *6*, 987–993.

Rougier, N., Bourc'his, D., Gomes, D.M., Niveleau, A., Plachot, M., Pàldi, A., and Viegas-Péquignot, E. (1998). Chromosome methylation patterns during mammalian preimplantation development. Genes Dev. *12*, 2108–2113.

Saitou, M., Kagiwada, S., and Kurimoto, K. (2012). Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. Development *139*, 15–31.

Sasaki, H., and Matsui, Y. (2008). Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat. Rev. Genet. *9*, 129–140.

Seisenberger, S., Peat, J.R., and Reik, W. (2013). Conceptual links between DNA methylation reprogramming in the early embryo and primordial germ cells. Curr. Opin. Cell Biol. *25*, 281–288.

Shen, L., Inoue, A., He, J., Liu, Y., Lu, F., and Zhang, Y. (2014). Tet3 and DNA replication mediate demethylation of both the maternal and paternal genomes in mouse zygotes. Cell Stem Cell *15*, 459–470.

Smith, Z.D., and Meissner, A. (2013). DNA methylation: roles in mammalian development. Nat. Rev. Genet. *14*, 204–220.

Smith, Z.D., Chan, M.M., Mikkelsen, T.S., Gu, H., Gnirke, A., Regev, A., and Meissner, A. (2012). A unique regulatory phase of DNA methylation in the early mammalian embryo. Nature *484*, 339–344.

Smith, Z.D., Chan, M.M., Humm, K.C., Karnik, R., Mekhoubad, S., Regev, A., Eggan, K., and Meissner, A. (2014). DNA methylation dynamics of the human preimplantation embryo. Nature *511*, 611–615.

Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell *153*, 910–918.

Wang, L., Zhang, J., Duan, J., Gao, X., Zhu, W., Lu, X., Yang, L., Zhang, J., Li, G., Ci, W., et al. (2014). Programming and inheritance of parental DNA methylomes in mammals. Cell *157*, 979–991.

Weyrich, A. (2012). Preparation of genomic DNA from mammalian sperm. Curr. Protoc. Mol. Biol. *Chapter 2*, 1–3.

White, J.K., Gerdin, A.K., Karp, N.A., Ryder, E., Buljan, M., Bussell, J.N., Salisbury, J., Clare, S., Ingham, N.J., Podrini, C., et al.; Sanger Institute Mouse Genetics Project (2013). Genome-wide generation and systematic phenotyping of knockout mice reveals new roles for many genes. Cell *154*, 452–464.

Wossidlo, M., Nakamura, T., Lepikhov, K., Marques, C.J., Zakhartchenko, V., Boiani, M., Arand, J., Nakano, T., Reik, W., and Walter, J. (2011). 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. Nat. Commun. *2*, 241.

Wu, S.C., and Zhang, Y. (2010). Active DNA demethylation: many roads lead to Rome. Nat. Rev. Mol. Cell Biol. *11*, 607–620.

Wu, H., and Zhang, Y. (2014). Reversing DNA methylation: mechanisms, genomics, and biological functions. Cell *156*, 45–68.